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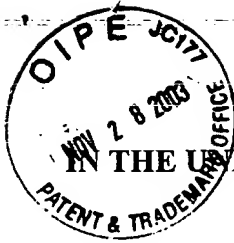
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 09/992,149
Applicant : Robert Brown, et al
Filed : 11/06/2001
TC/A.U. : 1645
Examiner : Minnifield, Nita M

Confirmation No. 5280

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Docket No. : 84077
Customer No. : 07380

DECLARATION PURSUANT TO 37 CFR § 1.131

We, Robert Brown, Warwick Kimmins and Bill Pohajdak hereby declare that:

1. We are the co-inventors of the subject matter of the pending claims of the above-identified patent application.
2. Prior to June 29, 2000, we conceived and reduced to practice the invention as described and claimed in the patent application in a NAFTA country, namely Canada, as evidenced by the following.
3. We previously conducted studies of immunocontraception of seals, using a vaccine composition in which a zona pellucida antigen was encapsulated in liposomes and the liposomes were suspended in oil. With this vaccine, we were able to effect immunocontraception in seals, through the use of a single administration of the vaccine. We realized that the vaccine formulation had unique properties of an unknown nature because no single administration vaccine that reduced mammalian fertility had been reported in the literature.
4. We had hypothesized that the unique vaccine formulation induced the production of antibodies with high affinity for the target antigen, namely the surface of the mammalian egg — i.e. the zona pellucida.
5. The generation of these high affinity antibodies occurred despite the fact that we had extracted the antigen from mammalian eggs by high temperature (80-90°C for 30 mins.). It is well known that subjecting proteins to this heat regime denatures the protein. Therefore, we decided to test the hypothesis that use of a vaccine formulation including and antigen encapsulated in liposomes, a carrier having a continuous oil phase, and an adjuvant, caused the production of antibodies with greater affinity for the native antigen despite the fact that denatured antigen was used for immunization.
6. One difficulty we faced in testing this hypothesis was that there was no easy method of

establishing that heat-extracted zona pellucida was denatured. To overcome this problem, we conducted experiments using alcohol dehydrogenase (ADH). Because ADH is an enzyme, unlike zona pellucida, loss of enzymatic activity could be used to demonstrate that the protein was denatured.

7. We denatured ADH (Sigma Chemical Company catalogue no. A-7011) using a combination of heat (100°C for 10 mins.) and treatment with mercaptoethanol (10% v/v in Tris buffer, 20 mM, pH 7.5). We vaccinated eight rabbits (two rabbits in each treatment group) with either native ADH or denatured ADH using conventional immunization or a vaccine composition in accordance with the invention. Conventional immunization used a primary inoculation given intramuscularly followed one month later by a booster intramuscular inoculation. The vaccine used for the primary inoculation contained native or denatured ADH (40 µg/dose) dissolved in saline (0.2 ml/dose) which was emulsified in Freund's complete adjuvant (0.2 ml/dose). The vaccine used for the booster inoculation contained native or denatured ADH (40 µg/dose) dissolved in saline (0.2 ml/dose) emulsified in Freund's incomplete adjuvant (0.2 ml/dose). For vaccination in accordance with the invention, a single inoculation was administered intramuscularly. The vaccine used contained native or denatured ADH (40 µg/dose) encapsulated in liposomes (0.04 gm phospholipon 90G and 0.004 gm cholesterol/dose) suspended in saline (0.2 ml/dose). The liposome suspension was emulsified in Freund's complete adjuvant (0.2 ml/dose).

8. Two months post immunization, the eight rabbits were bled. Anti-ADH antibodies raised by the vaccines were assayed for affinity to native ADH relative to denatured ADH. Exhibit A hereto is a copy of our lab notes concerning this experiment. The date deleted from Exhibit A is prior to June 29, 2000. The relevant data are those on the third page of the exhibit, wherein "Plate 5" is data for native ADH and "Plate 6" is data for denatured ADH. These data are more clearly presented in Table 1 below.

Table 1. Production of anti-ADH antibodies 2 months post-immunization by rabbits immunized with native or denatured ADH delivered using the invention or conventional immunization protocols.

| Rabbit ID | Immunization | | Titer (% reference serum) | |
|-----------|---------------|--------------|---------------------------|---------------|
| | Antigen | Delivery | Native ADH | Denatured ADH |
| 231 | native ADH | conventional | 7 | 4 |
| 232 | native ADH | conventional | 16 | 3 |
| 233 | native ADH | invention | 27 | 3 |
| 234 | native ADH | invention | 100 | 24 |
| 235 | denatured ADH | conventional | 6 | 5 |
| 236 | denatured ADH | conventional | 2 | 4 |
| 237 | denatured ADH | invention | 30 | 3 |
| 238 | denatured ADH | invention | 9 | 3 |

The reference serum is the 2 month post-immunization serum from rabbit 234 which was set at 100 % against native ADH.

9. The results of this experiment indicated that rabbits immunized with denatured ADH in accordance with the invention produced more antibodies directed against native ADH, than did rabbits immunized conventionally. This is clearly demonstrated in the data presented for rabbits 235 and 236 (i.e. denatured ADH administered by conventional immunization) versus rabbits 237 and 238 (i.e. denatured ADH in a vaccine composition of the invention). Rabbit ID numbers 237 and 238 produced antibodies having a much greater affinity to native ADH than did rabbits ID numbers 235 and 236.

10. These results supported our hypothesis that immunization in accordance with the invention results in generation of more antibodies directed against native epitopes of ADH, even in rabbits immunized with denatured ADH. These data indicated to us that vaccine compositions of the invention induced a conformational change in the heat-denatured ADH, changing it to a conformation closer to that of the native ADH enzyme, such that antibodies raised against vaccines of the invention better recognize native antigens.

11. To demonstrate that the results reported above were not unique to ADH, another antigen was studied, namely, porcine zona pellucida (pZP). pZP was heat extracted (80-90 °C for 30 minutes) from porcine oocytes to yield a soluble form of pZP called soluble intact zona pellucida (SIZP). SIZP consists of three glycoproteins termed ZPA, ZPB and ZPC. Together ZPB and ZPC account for 80 % of SIZP. We vaccinated grey seals with SIZP using conventional immunization

or a vaccine composition in accordance with the invention. Conventional immunization used a primary inoculation given intramuscularly followed one and two months later by booster intramuscular inoculations. The vaccine used for the primary inoculation contained SIZP (100 μ g/dose) dissolved in saline (0.5 ml/dose) which was emulsified in Freund's complete adjuvant (0.5 ml/dose). The vaccine used for the booster inoculations contained SIZP (100 μ g/dose) dissolved in saline (0.5 ml/dose) emulsified in Freund's incomplete adjuvant (0.5 ml/dose). For vaccination in accordance with the invention a single inoculation was administered intramuscularly. The vaccine used contained SIZP (100 μ g/dose) encapsulated in liposomes (0.1 gm phospholipon 90G and 0.01 gm cholesterol/dose) suspended in saline (0.5 ml/dose). The liposome suspension was emulsified in Freund's complete adjuvant (0.5 ml /dose).

12. One month after the last booster inoculation and monthly thereafter, grey seals immunized conventionally were bled. Three months after the single inoculation with vaccine and every other month thereafter, grey seals immunized according to the invention were bled. The time between the first inoculation and taking the first serum sample was the same for seals immunized conventionally or with the invention. Anti-SIZP antibodies raised by the vaccines were assayed for affinity to SIZP and four peptides that spanned essentially the entire length of the polypeptide chains of ZPB and ZPC that account for 80 % by weight of SIZP (see Figure 1 and Table 2).

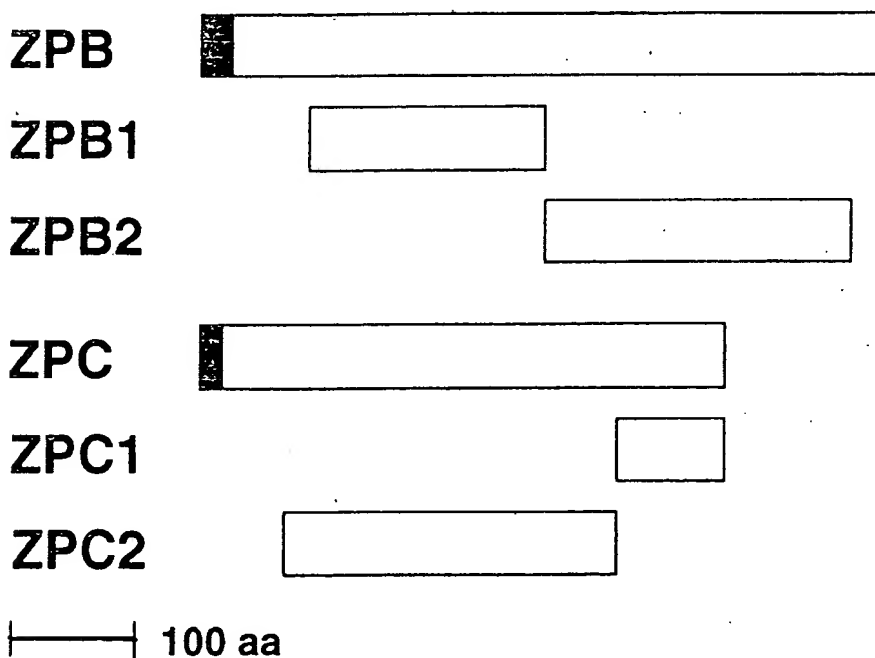


Figure 1. A diagrammatic presentation of the four peptides derived from ZPB and ZPC that span the length of the polypeptide chains of ZPB and ZPC. The areas of ZPB and ZPC shaded in black are the portions of ZPB and ZPC inserted in the oocyte membrane and therefore are unavailable to bind antibodies.

Table 2. Affinity of anti-SIZP antibodies for SIZP and four peptides derived from the ZPB and ZPC components of SIZP produced by grey seals immunized using the invention or conventional immunization protocols.

| Grey seal | Post-Immunization (months) | Delivery | Titer (% of reference serum) ¹ | | | | |
|-----------|-------------------------------|--------------|---|------|------|------|------|
| | | | SIZP | ZPB1 | ZPB2 | ZPC1 | ZPC2 |
| 1 | 3 | conventional | 100 | 30 | 44 | 59 | 41 |
| | 4 | | 100 | 71 | 70 | 83 | 63 |
| | 5 | | 100 | 99 | 129 | 120 | 136 |
| 77 | 3 | invention | 100 | 57 | 17 | 14 | 6 |
| | 5 | | 100 | 66 | 4 | 3 | 2 |
| | 7 | | 100 | 5 | 5 | 4 | 4 |
| 78 | 3 | invention | 100 | 62 | 17 | 5 | 8 |
| | 5 | | 100 | 63 | 5 | 5 | 3 |
| | 7 | | 100 | 13 | 7 | 10 | 9 |

¹ Titer measurements by ELISA placed 1 μ g substrate in each well of the microtiter plate, therefore, the maximum amount of antibody that could bind to the fragments is about 20 X's the amount of antibody that could bind to SIZP before saturation occurred. This is only relevant to the grey seal immunized conventionally which produced a lot of antibody specific for the fragments. The reference serum titer is the quantity of antibody bound by SIZP which was set at 100 % in all cases.

13. Exhibit B hereto is a copy of our lab notes concerning this further experiment. The date deleted from Exhibit B is prior to June 29, 2000. The relevant data are those concerning "Plate 3" on the third page of the exhibit. The information on page 2 of Exhibit B is reproduced above in Table 2. These results supported our hypothesis that immunization in accordance with the invention results in generation of more antibodies directed against the complete zona pellucida, that is, SIZP. In contrast, grey seals immunized conventionally produce more antibody directed against fragments of the antigen rather than structural elements of the antigen present only in the complete polypeptide chain. Several hypotheses can be proposed to explain these results; for example, structural elements may be protected by liposome delivery of antigens or liposome delivery may alter antigen processing by antigen presenting cells.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are

punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Nov. 14, 2003
Date

Robert Brown
Robert Brown

Nov 14, 2003
Date

Warwick Kimmins
Warwick Kimmins

Nov. 14, 2003
Date

B. Pohajdak
Bill Pohajdak

Exhibit A

(page 1)

Plate 1

% of Sacco

| | | |
|----|---------|------------------|
| 1 | Grey 1 | 10 |
| 2 | Grey 77 | 42 |
| 3 | 78 | 29 |
| 4 | 79 | 10 |
| 5 | 80 | 72 |
| 6 | 81 | 11 |
| 7 | 82 | 4 |
| 8 | 83 | 70 |
| 9 | 85 | 14 |
| 10 | 86 | 30 33 |
| 11 | Sacco | |
| 12 | Blk. | |

Plate 2.

% of Sacco

| | | |
|----|----------|----|
| 1 | Grey 87 | 77 |
| 2 | " 88 | 82 |
| 3 | Harp 151 | 8 |
| 4 | " 153 | 8 |
| 5 | " 154 | 1 |
| 6 | " 156 | 46 |
| 7 | " 157 | 3 |
| 8 | " 158 | 4 |
| 9 | " 159 | 5 |
| 10 | " 162 | 26 |
| 11 | Sacco | |
| 12 | Blk. | |

Plate 3

| | | % of Saccs |
|----|------------|------------|
| 1 | Rabbit 220 | 102 |
| 2 | 221 | 106 |
| 3 | 202 | 152 |
| 4 | 212 | 135 |
| 5 | 213 | 115 |
| 6 | 214 | 81 |
| 7 | 241 | 0.4 |
| 8 | 242 | 0.5 |
| 9 | 243 | 0.5 |
| 10 | Grey 139 | 91 |
| 11 | Sapco | |
| 12 | BLK | |

Plate 4 Rabbit anti-hyp serum

| | | % of Saccs |
|----|----------|------------|
| 1 | Harp 151 | 13 |
| 2 | 153 | 9 |
| 3 | 154 | 1 |
| 4 | 156 | 47 |
| 5 | 157 | 5 |
| 6 | 158 | 6 |
| 7 | 159 | 5 |
| 8 | 162 | 43 |
| 9 | Sapco | |
| 10 | BLK | |
| 11 | | |
| 12 | | |

Rabbit Anti-hyp serum (1/100)

4:50 → 5:50

Protein A 5:50 → 6:20

Plate 5 Native ADH

% of reference serum

1
2
3
4
5
6
7
8
9
10
11
12Rabbit 231
232
233
234
235
236
237
2387
16
27
150
6
2
30
9

Plate 6

Denatured ADH

% of reference serum

1
2
3
4
5
6
7
8
9
10
11
12Rabbit 231
232
233
234
235
236
237
2384
3
3
24
5
4
3
3Ag
Deleters
AL
Protein A1 PM → 2 PM
2:45 → 3 PM
3:45 → 4:45 PM
5:15 → 5:45 PM

Exhibit B
page 1

Plate 1

| | | | | | |
|----|-------|----|-----|---|----|
| 1 | 77 | 77 | +69 | = | 73 |
| 2 | 78 | 51 | +59 | = | 55 |
| 3 | 79 | 9 | +20 | = | 10 |
| 4 | 80 | 84 | +77 | = | 81 |
| 5 | 81 | 9 | | | |
| 6 | 82 | 6 | | | |
| 7 | 83 | 72 | | | |
| 8 | 85 | 18 | | | |
| 9 | 86 | 53 | | | |
| 10 | 87 | 67 | | | |
| 11 | Sacco | | | | |
| 12 | Blk. | | | | |

Plate 2

| | | | | | |
|----|-------|-----|------|---|------|
| 1 | 88 | 100 | +86 | = | 93% |
| 2 | 139 | 120 | +139 | = | 130% |
| 3 | 61 | 2 | +4 | = | 3% |
| 4 | 88 | 86 | | | |
| 5 | 139 | 139 | | | |
| 6 | 61 | 4 | | | |
| 7 | 77 | 69 | | | |
| 8 | 78 | 59 | | | |
| 9 | 79 | 20 | | | |
| 10 | 80 | 77 | | | |
| 11 | Sacco | | | | |
| 12 | Blk. | | | | |

Epitope mapping of grey seal anti-SIZP sera with denatured fragments of ZP 3 α and ZP 3 β produced in bacteria.

Crossreactivity relative to SIZP (%)

| Seal ID ¹ | Post-immunization (months) | ZP 3 α fragments | | ZP 3 β fragments | | Total |
|----------------------|----------------------------|-------------------------|------------|------------------------|-----------|-------|
| | | α | | β | | |
| | | α 1 | α 2 | β 1 | β 2 | |
| 1 | 3 | 30 | 44 | 59 | 41 | 174 |
| 1 | 4 | 71 | 70 | 83 | 63 | 287 |
| 1 | 5 | 99 | 129 | 120 | 136 | 484 |
| 77 | 3 | 57 | 17 | 14 | 6 | 96 |
| 77 | 5 | 66 | 4 | 3 | 2 | 75 |
| 77 | 7 | 5 | 5 | 4 | 4 | 18 |
| 77 | 9 | 7 | 3 | 3 | 1 | 14 |
| 77 | 11 | 13 | 6 | 3 | 4 | 26 |
| 77 | 13 | 6 | 3 | 2 | 2 | 13 |
| 77 | 15 | 7 | 6 | 2 | 1 | 16 |
| 77 | 17 | 12 | 8 | 7 | 7 | 34 |
| 77 | 19 | 8 | 7 | 5 | 4 | 24 |
| 78 | 3 | 62 | 17 | 5 | 8 | 92 |
| 78 | 5 | 63 | 5 | 5 | 3 | 76 |
| 78 | 7 | 13 | 7 | 10 | 9 | 39 |
| 78 | 9 | 10 | 7 | 7 | 6 | 30 |
| 78 | 11 | 10 | 7 | 9 | 7 | 33 |
| 78 | 13 | 8 | 4 | 4 | 5 | 21 |
| 78 | 15 | 11 | 8 | 10 | 8 | 37 |
| 78 | 17 | 14 | 6 | 11 | 11 | 42 |
| 78 | 19 | 12 | 12 | 6 | 7 | 37 |

¹ Grey seal 1 was immunized using FCA/FIA as adjuvant. Grey seals 77 and 78 were immunized using FCA as adjuvant with liposome delivery. The porcine zona pellucida (ZP) fragments were denatured with 4 M guanidinium isothiocyanate.

Plate 3

1 Q1

2 Q2

3 Q1

4 Q2

5 2P3

6 512P

7

8

9

10

11

12

10

71

70

83

63

48

9

287%

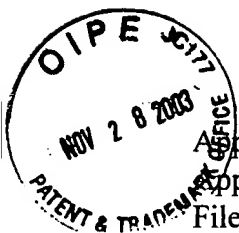
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Ag
Gelatin
Alb
Protein A

10:50 → 11:50

1:20 → 2:20

3:05 → 3:35



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

App. No. : 09/992,149
Applicant : Robert Brown, et al
Filed : 11/06/2001
TC/A.U. : 1645
Examiner : Minnifield, Nita M

Confirmation No. 5280

Docket No. : 84077
Customer No. : 07380

DECLARATION PURSUANT TO 37 CFR § 1.132

I, Robert Brown, hereby declare that:

1. I am an inventor in the above-identified application.
2. I have conducted an experiment comparing antibody production by rabbits immunized with heat denatured pertussis toxin using conventional immunization protocols versus vaccine compositions of the present invention.

3. Materials and Methods

Pertussis toxin (Calbiochem, La Jolla, CA; catalogue # 516562) was denatured by dissolving the toxin (50 µg) in saline (1.25 ml) then placing the solution in water at 100 C for 15 minutes. For conventional immunization, rabbits were immunized intramuscularly using heat denatured pertussis toxin (5 µg/dose) with alum adjuvant (0.125 ml ImjectAlum/dose, Pierce Chemical Co. cat # 77161). The pertussis toxoid/alum complex in saline (0.125 ml/dose) was emulsified in low viscosity mineral oil (0.225 ml/dose) containing mannide oleate (0.025 ml /dose). For immunization using the invention, rabbits were immunized intramuscularly using heat denatured pertussis toxoid (5 µg/dose) with alum adjuvant (0.125 ml/dose) encapsulated in liposomes containing soybean lecithin (0.1 gm/dose) and cholesterol (0.01 gm/dose). The liposomes were suspended in saline (0.125 ml/dose) and the suspension emulsified in low viscosity mineral oil (0.225 ml/dose) containing mannide oleate (0.025 ml/dose).

4. Native pertussis toxin is known to promote apoptosis of macrophages. Therefore, native pertussis toxin should inhibit antigen processing and decrease the immune response. In contrast, heat denatured pertussis toxin (pertussis toxoid) should be inactive and a normal immune response should be observed against the pertussis toxoid. This was observed when conventional immunization protocols were used, as shown in the table below.

Table 15. Production of anti-pertussis toxin antibodies by rabbits


| Rabbit ID | Delivery | Anti-pertussis toxin titer Post-immunization (Months) | | | |
|-----------|--------------|--|-----|-----|-----|
| | | 0 | 1 | 2 | 3 |
| 138 | Conventional | 0 | 66 | 65 | 153 |
| 139 | Conventional | 0 | 33 | 69 | 107 |
| 140 | Conventional | 0 | 85 | 53 | 25 |
| 141 | Conventional | 0 | 28 | 44 | 15 |
| 142 | Conventional | 0 | 19 | 41 | 20 |
| 143 | Conventional | 0 | 100 | 106 | 165 |
| 144 | Invention | 0 | 0 | 2 | 22 |
| 145 | Invention | 0 | 4 | 13 | 27 |
| 146 | Invention | 0 | 3 | 19 | 30 |
| 147 | Invention | 0 | 0 | 2 | 3 |
| 148 | Invention | 0 | 2 | 17 | 17 |
| 149 | Invention | 0 | 1 | 3 | 5 |
| 150 | Invention | 0 | 0 | 2 | 5 |

In contrast, when rabbits were immunized with the same pertussis toxoid using a vaccine composition of the invention, the immune response was greatly reduced.

5. I believe that the invention induced a conformational change in the heat denatured pertussis toxin (pertussis toxoid) to a conformation closer to a native pertussis toxin. This made it similar enough to native pertussis toxin to cause apoptosis of macrophages, thereby inhibiting an immune response.

6. This is further evidence that vaccine compositions of the instant invention restore the native three-dimensional conformation of the antigen, such that antibodies raised against the vaccines of the invention better recognize native antigens.

7. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.


Robert Brown

Oct. 31, 2003
Date